VERIFIED TRANSLATION OF PCT 31525/99

helianthin

IN THE MATTER OF an Australian
Application corresponding to
PCT Application PCT/FR99/00843

I, Abraham SMITH DipIng, DipDoc,
translator to RWS Group plc, of Europa House, Marsham Way, Gerrards Cross, Buckinghamshire, England, do solemnly and sincerely declare that I am conversant with the English and French languages and am a competent translator thereof, and that to the best of my knowledge and belief the following is a true and correct translation of the PCT Application filed under No. PCT/FR99/00843.

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A. SMITH
For and on behalf of RWS Group plc

helianthine (Helianthus tuberosus)

pp 22 patents on plant transformation
pp 23, 39 fungal activity
mentioned in good
pp 40 yeast activity
plant transformation pp 40 →
pp 52 expression in transgenic tobacco – 4/8 plants express
pp 53 quantifications / sprays, etc
pp 57 stability – incubation with plant extracts
pp 58, 66 ingredients + activity
pp 61 animal toxicity – none

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(54) Titre: GENE CODANT POUR L'HELIOMICINE ET SON UTILISATION

(57) Abstract

The invention concerns heliomicine, a DNA sequence coding for heliomicine, a vector containing it for transforming a host organism and the transformation method. The invention concerns heliomicine as medicine in particular for treating fungal infections. More particularly it concerns the transformation of plant cells and plants, the heliomicine produced by the transformed plants ensuring their resistance to diseases, in particular diseases of fungal origin.

(57) Abrégé

La présente invention a pour objet l'héliomicine, une séquence d'ADN codant pour l'héliomicine, un vecteur la contenant pour la transformation d'un organisme hôte et le procédé de transformation. L'invention concerne l'utilisation de l'héliomicine à titre de médicament, en particulier pour le traitement des infections fongiques. L'invention concerne plus particulièrement la transformation des cellules végétales et des plantes, l'héliomicine produite par les plantes transformées leur conférant une résistance aux maladies, en particulier d'origine fongique.

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GENE ENCODING HELIOMICINE AND ITS USE

The subject of the present invention is a new cysteine-rich peptide called heliomicine, its use as a medicament and the compositions containing it, a DNA sequence encoding this peptide, a vector containing it for the transformation of a host organism and the method of transforming the said organism.

The invention relates more particularly to the transformation of plant cells and of plants, the heliomicine produced by the transformed plants conferring on them resistance to diseases, in particular of fungal origin.

There is currently an increasing need to make plants resistant to diseases, in particular fungal diseases, in order to reduce or even avoid having to use treatments with antifungal protection products, in order to protect the environment. One means of increasing this resistance to diseases consists in transforming plants so that they produce substances capable of providing their defence against these diseases.

In the field of human health, opportunistic fungal infections exist for which no truly effective treatment currently exists. In particular, this is the case for certain serious invasive mycoses which affect hospital patients whose immune system is suppressed following a transplant, a chemotherapy or HIV

responsible for plant diseases and the fungi of human or animal pathology. After having first synthesized the gene for heliomicine, it was also found that it could be inserted into a host organism, such as a yeast or a plant, so as to express heliomicine and either produce purified or nonpurified heliomicine, or confer on the said host organism properties of resistance to fungal diseases, providing a particularly advantageous solution to the problems set out above.

The subject of the invention is therefore first heliomicine, its use as a medicament or in agrochemistry for the protection of plants, the compositions comprising it, a nucleic acid fragment encoding heliomicine, a chimeric gene comprising the said fragment encoding heliomicine as well as heterologous regulatory elements at the 5' and 3' positions which can function in a host organism, in particular in yeasts or plants and a vector for transforming the host organisms containing this chimeric gene, and the transformed host organism. It also relates to a transformed plant cell containing at least one nucleic acid fragment encoding heliomicine and a plant resistant to diseases containing the said cell, in particular which is regenerated from this cell. It finally relates to a method of transforming plants to make them resistant to diseases into which a gene encoding heliomicine is inserted by means of an

acids.

Advantageously, Xad represents the following peptide sequence -Lys-Xad'-Xad"-Gly-His-, in which Xad' represents a peptide residue of 1 basic amino acid and Xad" represents a peptide residue comprising from 0 to 5 amino acids, preferably 5.

Basic amino acids are understood to mean more particularly according to the invention the amino acids chosen from lysine, arginine or homoarginine.

Preferably, Xad represents the following peptide sequence -Lys-Arg-Arg-Gly-Tyr-Lys-Gly-Gly-His- or Leu-Leu-Arg-Gly-Tyr-Lys-Gly-Gly-His-.

According to another preferred embodiment of the invention, Xac comprises at least one acidic amino acid, preferably one.

Advantageously, Xac represents the following peptide sequence -Asn-Xac'-Xac"-, in which Xac' represents a peptide residue of 1 amino acid, and Xac" represents a peptide residue of 1 acidic amino acid.

Acidic amino acid is understood to mean according to the invention any amino acid comprising on a side chain an organic acid function, more particularly a carboxylic acid preferably chosen from glutamic acid (Glu) or aspartic acid (Asp).

Preferably, Xac represents the following peptide sequence -Asn-Gly-Glu- or Ala-Ala-Glu-.

Advantageously,

represents the following amino acid -Trp- and/or Xag represents the following peptide sequence -Glu-Thr-OH or -Arg-Thr-OH.

According to a more preferred embodiment of the invention, the heliomicine is the peptide represented with its coding sequence by the sequence identifier No. 2 (SEQ ID NO 2). The same sequence is described, corresponding to amino acids 6 to 49 of the sequence identifier No. 1 (SEQ ID NO 1) with a different coding sequence.

The NH_2-terminal residue may exhibit a post-translational modification, for example an acetylation, likewise the C-terminal residue may exhibit a post-translational modification, for example an amidation.

Peptide sequence comprising essentially the peptide sequence of general formula (I) is understood to mean not only the sequences defined above, but also such sequences comprising at either of their ends, or at both ends, peptide residues necessary for their expression and targeting in a host organism. Host organism is understood to mean any organism comprising at least one cell, whether microorganisms, in particular a yeast or a bacterium, or alternatively plant cells or alternatively higher organisms such as plants.

This may be in particular a "peptide-heliomicine" fusion peptide whose cleavage by the

the direction of transcription, a sequence encoding a transit peptide of a plant gene encoding a plastid localization enzyme, a portion of sequence of the N-terminal mature part of a plant gene encoding a plastid localization enzyme, and then a sequence encoding a second transit peptide of a plant gene encoding a plastid localization enzyme, as described in application EP 0 508 909.

As transit peptide useful according to the invention, there may be mentioned in particular the signal peptide of the tobacco PR-1α gene described by Cornelissen *et al.*, represented with its coding sequence by the sequence identifier No. 2, in particular when heliomicine is produced by plant cells or plants, or the precursor of factor Mat α1 when heliomicine is produced in yeasts.

The fusion peptide "MFα1/heliomicine" with the five residues of the propeptide of factor MFα1 (Ser-Leu-Asp-Lys-Arg), which are situated at the N-terminal position, and its coding sequence are part of the present invention, described in particular by the sequence identifier No. 1 (SEQ ID NO 1), corresponding to amino acids 1 to 49.

The "PR-1α signal peptide-heliomicine" fusion peptide and its coding sequence are also part of the present invention, described in particular by the sequence identifier No. 3 (SEQ ID NO 3).

composition may be a cosmetic composition and in this case the appropriate vehicle is cosmetically acceptable (suitable in addition for application to the skin or the exoskeleton), or a pharmaceutical composition for a therapeutic use and in this case the appropriate vehicle is pharmaceutically acceptable, appropriate for administration of heliomicine by the topical route per os or by injection, or alternatively an agrochemical composition and in this case the appropriate vehicle is agrochemically acceptable, appropriate for application to plants or in the vicinity of plants, without damaging them.

The present invention also relates to a nucleic acid fragment, in particular DNA, natural or synthetic, encoding the heliomicine defined above, including the "peptide-heliomicine" fusion peptide defined above. It may be according to the invention a fragment which is synthesized or which is isolated from the lepidepteron *Heliothis*, or alternatively a derived fragment, suitable for the expression of heliomicine in the host organism where the peptide will be expressed. The nucleic acid fragment may be obtained according to standard isolation and purification methods, or alternatively by synthesis according to the customary methods of successive hybridizations of synthetic oligonucleotides. These techniques are in particular described by Ausubel *et al.*

sequence described by the sequence identifiers Nos. 1, 2 or 3 and encoding heliomicine or the "peptide-heliomicine" fusion peptide. These modifications may be obtained according to the customary mutation techniques, or alternatively by choosing the synthetic oligonucleotides used in the preparation of the said sequence by hybridization. In the light of the multiple combinations of nucleic acids which may lead to the expression of the same amino acid, the differences between the reference sequence described by the sequence identifiers Nos. 1, 2 or 3 and the corresponding homologue may be substantial, all the more so since small-sized DNA fragments are involved which can be produced by chemical synthesis. Advantageously, the degree of homology will be at least 70% compared with the reference sequence, preferably at least 80%, more preferably at least 90%. These modifications are generally neutral, that is to say that they do not affect the primary sequence of the resulting heliomicine or fusion peptide.

The present invention also relates to a chimeric gene (or expression cassette) comprising a coding sequence as well as heterologous regulatory elements at the 5' and 3' positions capable of functioning in a host organism, in particular plant cells or plants, the coding sequence comprising at least one DNA fragment encoding heliomicine or the

sequences, transcription activators, terminator sequences, including start and stop codons. The means and methods for identifying and selecting the regulatory elements are well known to persons skilled in the art.

For the transformation of microorganisms such as yeasts or bacteria, the regulatory elements are well known to persons skilled in the art, and comprise in particular promoter sequences, transcription activators, transit peptides, terminator sequences and start and stop codons.

To direct the expression and the secretion of the peptide in the yeast culture medium, a DNA fragment encoding heliomicine is integrated into a shuttle vector which comprises the following elements:

- markers which make it possible to select the transformants. Preferably, the ura-3 gene is used for yeast and the gene which confers resistance to ampicilline for *E. coli*,
- a nucleic sequence allowing the replication (replication origin) of the plasmid in yeast. Preferably, the replication origin of the yeast 2ì plasmid is used,
- a nucleic sequence allowing the replication (replication origin) of the plasmid in *E. coli*,
- an expression cassette consisting

(1) of a promoter regulatory sequence. Any

The transformation of microorganisms makes it possible to produce heliomicine on a larger scale. The present invention therefore also relates to a method of preparing heliomicine, comprising the steps of culturing a transformed microorganism comprising a gene encoding heliomicine as defined above in an appropriate culture medium, followed by the extraction and total or partial purification of the heliomicine obtained.

Preferably, during the extraction of the heliomicine produced by yeasts, the yeasts are removed by centrifugation and the culture supernatant is placed in contact with an acidic solution which may be a solution of an inorganic or organic acid, such as for example hydrochloric acid or acetic acid. The extract obtained is then centrifuged at cold temperature at a speed of 4000 to 10,000 rpm at 4°C for 30 to 60 min.

The purification of heliomicine may be preceded by a step of fractionation of the supernatant obtained following the extraction step. Preferably, during the fractionation step, the extract is deposited on the reversed phase in order to carry out a solid phase extraction. The washing of the molecules which are soluble in water is carried out with a dilute acidic solution and the elution of the hydrophobic molecules with an appropriate eluant. Good results are obtained with trifluoroacetic acid for the washing and an eluant containing increasing quantities of

sequence in plants, it is possible to use any promoter sequence of a gene which is naturally expressed in plants, in particular a promoter of bacterial, viral or plant origin such as, for example, that of a gene for the small subunit of ribulose-biscarboxylase/oxygenase (RuBisCO) or of a plant virus gene such as, for example, that of the cauliflower mosaic (19S or 35S CAMV), or a promoter which is inducible by pathogens such as the tobacco PR-1α, it being possible to use any known suitable promoter. Preferably, a promoter regulatory sequence is used which promotes the overexpression of the coding sequence constitutively or induced by attack by a pathogen, such as for example that comprising at least one histone promoter as described in application EP 0,507,698.

According to the invention, it is also possible to use, in combination with the promoter regulatory sequence, other regulatory sequences which are situated between the promoter and the coding sequence, such as transcription activators (enhancer), such as for example the translation activator of the tobacco mosaic virus (TMV) which is described in application WO 87/07644, or of the tobacco etch virus (TEV) which is described by Carrington & Freed.

As polyadenylation or terminator regulatory sequence, there may be used any corresponding sequence of bacterial origin, such as for example the

replication and expression. Preferably, the vector for transforming plant cells or plants according to the invention is a plasmid.

The subject of the invention is also a method of transforming host organisms, in particular plant cells by integration of at least one nucleic acid fragment or a chimeric gene as defined above, which transformation may be obtained by any appropriate known means widely described in the specialized literature and in particular the references cited in the present application, more particularly using the vector according to the invention.

A series of methods consists in bombarding cells, protoplasts or tissues with particles to which DNA sequences are attached. Another series of methods consists in using, as means of transfer into plants, a chimeric gene inserted into an *Agrobacterium tumefaciens* Ti or *Agrobacterium rhizogenes* Ri plasmid.

Other methods may be used, such as microinjection or electroporation, or alternatively direct precipitation by means of PEG.

Persons skilled in the art will make the choice of the appropriate method according to the nature of the host organism, in particular of the plant cell or of the plant.

The subject of the present invention is also the host organisms, in particular plant cells or

encoding heliomicine may be integrated with the main objective of producing plants resistant to the said diseases, heliomicine being effective against fungal diseases such as those caused by *Cercospora*, in particular *Cercospora beticola*, *Cladosporium*, in particular *Cladosporium herbarum*, *Fusarium*, in particular *Fusarium culmorum* or *Fusarium graminearum*, or by *Phytophthora*, in particular *Phytophthora cinnamomi*.

The chimeric gene may also comprise, and advantageously, at least one selectable marker, such as one or more genes for tolerance to herbicides.

The DNA sequence encoding heliomicine may also be integrated as a selectable marker during the transformation of plants with other sequences encoding other peptides or proteins of interest, such as for example genes for tolerance to herbicides.

Such genes for tolerance to herbicides are well known to persons skilled in the art and are in particular described in patent applications EP 115,673, WO 87/04181, EP 337,899, WO 96/38567 or WO 97/04103.

Of course the transformed cells and plants according to the invention may comprise, in addition to the sequence encoding heliomicine, other heterologous sequences encoding proteins of interest such as other additional peptides which are capable of conferring on the plant resistance to other diseases of bacterial or

The present invention finally relates to a method of cultivating transformed plants according to the invention, the method consisting in planting the seeds of the said transformed plants in a plot of a field appropriate for cultivating the said plants, in applying to the said plot of the said field an agrochemical composition, without substantially affecting the said seeds or the said transformed plants, then in harvesting the cultivated plants when they arrive at the desired maturity and optionally in separating the seeds from the harvested plants.

Agrochemical composition is understood to mean according to the invention any agrochemical composition comprising at least one active product having one of the following activities: herbicide, fungicide, bactericide, virucide or insecticide.

According to a preferred embodiment of the method of cultivation according to the invention, the agrochemical composition comprises at least one active product having at least one fungicidal and/or bactericidal activity, more preferably exhibiting an activity which is complementary to that of the heliomicine produced by the transformed plants according to the invention.

Product exhibiting an activity which is complementary to that of heliomicine is understood to mean according to the invention a product exhibiting a

1-2 Preparation of the plasma

The haemolymph (about 30 µl per larva) was collected by excision of an abdominal appendage and collected in 1.5-ml polypropylene microcentrifuge tubes cooled on ice and containing aprotinin as protease inhibitor (20 µg/ml final concentration) and phenylthiourea as melanization inhibitor (final concentration of 20 µm). The haemolymph (2 ml) thus collected from the immunized larvae was centrifuged at 14,000 g for 1 min at 4°C in order to remove the haemocytes. The haemolymph, free of blood cells, was stored at -20°C up to its use.

1-3 Acidification of the plasma

After rapid thawing, the *H. virescens* plasma was acidified to pH 3 with a 1% trifluoroacetic acid solution. The extraction, under acidic conditions, of the peptide was carried out for 30 min, with gentle stirring, on an ice-cold bath. The extract obtained was then centrifuged at 4°C for 30 min at 10,000 g.

1-4 Purification of the peptides

a) Prepurification by solid phase extraction

A quantity of extract equivalent to 2 ml of haemolymph was deposited on a reversed-phase support, as marketed in the form of a cartridge (Sep-Pak™ C18, Waters Associates), equilibrated with acidified water (0.05% TFA). The hydrophilic molecules were removed by a simple wash with acidified water. The elution of the

manually, monitoring the variation of the absorbance at 225 nm and 254 nm. The fractions collected were dried under vacuum, reconstituted with ultrapure water and analysed for their antifungal activity under the conditions described below.

- **third step**: the antifungal fraction containing the peptide was purified to homogeneity on a Narrowbore Delta-Pak™ $HPIC_{18}$ reversed-phase column (Waters Associates, 150 × 2.2 mm) using a biphasic linear gradient of acetonitrile from 2% to 24% over 10 min and from 24 to 44% over 100 min in 0.05% TFA with a constant flow rate of 0.25 ml/min at a controlled temperature of 30°C. The fractions were collected manually, monitoring the variation of the absorbance at 225 nm. The fractions collected were dried under vacuum, reconstituted with filtered ultrapure water and analysed for their antifungal activity.

Example I.2: structural characterization of the peptide

2-1 Verification of purity by zonal capillary electrophoresis

The purity of the antifungal peptide was verified by zonal capillary electrophoresis on a 270-HT model (*PE*Applied Biosystems division of Perkin Elmer). 1 nl of a 50 ìM solution of purified peptide was injected with the aid of a vacuum into a silica capillary (72 cm × 50 ìm) and the analysis was carried out in a 20 mM citrate buffer at pH 2.5. The

were calibrated externally with a standard mixture of peptides of known m/z, respectively 2199.5 Da, 3046.4 Da and 4890.5 Da. The various products to be analysed were deposited on a thin layer of α-cyano-4-hydroxycinnamic acid crystals which is obtained by rapid evaporation of a solution saturated with ethanol. After drying under a moderate vacuum, the samples were washed with a drop of 0.1% trifluoroacetic acid before being introduced into the mass spectrum.

2-4 Sequencing by Edman degradation

The automated sequencing by Edman degradation of the native peptide, of the S-pyridylethylated peptide and of the various fragments obtained after the various proteolytic cleavages and the detection of the phenylthiohydantoin derivatives were carried out on an ABI473A sequencer (*PE*Applied Biosystems division of Perkin Elmer).

2-5 Proteolytic cleavages

- Confirmation of the peptide sequence in the C-terminal region

200 pmol of reduced and S-pyridylethylated peptide were incubated in the presence of 5 pmol of endoproteinase-Lys-C (*Acromobacter* protease I, specific cleavage of the lysine residues on the C-terminal side, Takara, Otsu) according to the conditions recommended by the supplier (10 mM Tris-HCl, pH 9, in the presence of 0.01% Tween 20). After stopping the reaction with 1%

All the techniques used below are standard laboratory techniques. The detailed protocols for these techniques have been described in particular in Ausubel *et al.*

Example II-1: Assembling of the synthetic gene

Assembling was carried out using 6 synthetic oligonucleotides encoding the 44 amino acids of heliomicine preceded by the 5 C-terminal amino acids of the pre-pro sequence of factor α1 (Mfα1) of the yeast. The oligonucleotides represented in Figure 1 were chosen taking into account the preferential codons used by S. cerevisiae.

The assembling took place in several steps:

- oligonucleotides 2 to 5 were phosphorylated at their 5' ends by the action of polynucleotide kinase (New England Biolabs);

- oligonucleotides 1 to 6 were mixed, heated to 100·C and hybridized by slowly reducing the temperature to 25·C over 3 hours;

- the hybridized oligonucleotides were subjected to a treatment with T4 bacteriophage ligase (New England Biolabs) for 15 hours at 15·C;

- the DNA unit resulting from the hybridization of the oligonucleotides which is represented in Figure 1, flanked by the HinDIII and BglII restriction sites, was inserted into the plasmid pBluescript SK+ (Stratagene) digested with the enzymes

Invert. Reprod. Dev. 21, pp 15-24) was transformed with the plasmid pSEA2. The transformants were selected at 29°C on a selective YNBG medium (0.67% yeast nitrogen base, 2% glucose), supplemented with 0.5% of casamino acids and containing no uracil. After transformation, several yeast clones, selected for the ura+ character, were cultured for 48 h at 29°C in 50 ml of selective medium. After centrifugation (4000 g, 30 min, 4°C), the supernatant was acidified to pH 3.5 with acetic acid, before being deposited on a Sep-Pak™ C_{18} cartridge (Waters Associates) equilibrated with acidified water (0.05% TFA). The various proteins bound to the cartridge were eluted with solutions of 0.05% TFA containing increasing percentages of acetonitrile.

The 40% fraction, exhibiting an antifungal activity, was analysed by HPLC on an Aquapore RP-300 C_8 reversed-phase analytical column (Brownlee™, 220 × 4.6 mm, 300 Å), using a linear gradient of acetonitrile from 2% to 40% over 80 min in 0.05% TFA with a constant flow rate of 0.8 ml/min. The fractions were collected manually by monitoring the variation in absorbance at 225 nm and 254 nm. The fractions collected were dried under vacuum, reconstituted with ultrapure water and analysed for their antifungal activity under the conditions described in Example III. The structural characterization of the peptide was carried out as described in Example I.2.

250 × 10 mm), using a linear gradient of NaCl from 0% to 100% over 90 min in 25 mM ammonium acetate, pH 3.4 with a constant flow rate of 2 ml/min. The fractions collected were dried under vacuum, reconstituted with ultrapure water and analysed for their antifungal activity under the conditions described below.

- second step of purification by HPLC: the heliomicine was purified to homogeneity by chromatography on an Aquapore RP-300 C_8 semipreparative reversed-phase column (Brownlee™, 220 × 7 mm, 300 Å), using a linear gradient of acetonitrile from 2% to 40% over 80 min in 0.05% TFA with a constant flow rate of 2 ml/min.

Example III: Test of activity in vitro: measurement of the antifungal activity by microspectrophotometry

This methodology was used to test for the antifungal molecules during the various purification steps, for the determination of the activity spectrum of the peptide and for the determination of the minimum inhibitory concentration (MIC) at which the peptide was active. The MIC was expressed as the concentration range [a] - [b] where [a] was the minimum concentration where the start of growth is observed and [b] the concentration for which no growth was observed. Examples of the specific activity of heliomicine, against filamentous fungi and yeasts, are given in Tables 1 and 2.

activity against filamentous fungi are presented in Table 1 below.

Table 1: activity of heliomicine against filamentous fungi

Fungi	MIC of heliomicine (ìM)
Neurospora crassa	0.1-0.2
Fusarium culmorum	0.2-0.4
Fusarium oxysporum	1.5-3
Nectria haematococca	0.4-0.8
Trichoderma viride	1.5-3
Aspergillus fumigatus	6-12.5

Example III-2: Test for detection of activity against yeasts

The various yeast strains were incubated in a "Sabouraud" type culture medium and incubated at 30°C for 24 h with gentle stirring. The test sample (10 ìl) was deposited in microtitre plate wells to which there were added 90 ìl of a dilute yeast culture whose density was adjusted to OD 600 = 0.001. Growth was evaluated by measuring the absorbance at 600 nm with the aid of a spectrophotometric microtitre plate reader.

- yeasts tested: *Candida albicans, C. glabrata, C. tropicalis, C. krusei, C. inconspicua, Cryptococcus neoformans, Cryp. albidus, Saccharomyces cerevisiae* (gift from Dr H. Koenig, Hôpital civil,

al.

Example IV-1: Construction of the chimeric genes for the transformation of plants

pRPA-MD-P: Creation of a plasmid containing the signal peptide of the tobacco PR-1α gene

The two complementary synthetic oligonucleotides Oligo 7 and Oligo 8 below are hybridized at 65°C for 5 minutes and by slow reduction of the temperature to 30°C over 30'.

Oligo 7: 5' GCGTCGACGC GATGGGTTTC GTGCTTTTCT CTCAGCTTCC ATCTTTCCTT CTTGTGTCTA CTCTTCTTCT TTTCC 3'

Oligo 8: 5' TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA GAAGAGTAGA CACAAGAAGG AAAGATGGAA GC 3'

After hybridization between Oligo 7 and Oligo 8, the DNA remaining single-stranded serves as template for the Klenow fragment of *E. coli* polymerase I (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the 3' end of each oligo. The double-stranded oligonucleotide obtained is then digested with the restriction enzymes SacII and NaeI and cloned into the plasmid pBS II SK(-) (Stratagene) digested with the same restriction enzymes. A clone is then obtained which comprises the region encoding the signal peptide of the tobacco PR-1α

NcoI restriction sites at the N-terminal end and the ScaI, SacII and BamHI restriction sites at the C-terminal end (SEQ ID NO 3).

pRPA-RD-239: Creation of a vector for expression in plants comprising the sequence encoding the PR-1α-heliomicine fusion protein

The plasmid pRTL-2 GUS, derived from the plasmid pUC-19, was obtained from Dr Jim Carrington (Texas A&M University, not described). This plasmid, whose schematic structure is represented in Figure 2, contains the duplicated CaMV 35S promoter isolated from the cauliflower mosaic virus (CaMV 2x35S promoter; Odell *et al.*, 1985) which directs the expression of an RNA containing the tobacco etch virus 5' untranslated sequence (TEV 5' UTR; Carrington & Freed, 1990), the *E. coli* â-glucoronidase gene (GUS Jefferson *et al.*, 1987) followed by the CaMV 35S RNA polyadenylation site (CaMV polyA; Odell *et al.*, 1985).

The plasmid pRTL-2 GUS is digested with the restriction enzymes NcoI and BamHI and the large DNA fragment is purified. The plasmid pRPA-PS-PR1α-helio is digested with the restriction enzymes NcoI and BamHI and the small DNA fragment containing the region encoding the PR-1α-heliomicine fusion protein is purified. The two purified DNA fragments are then ligated together into a cassette for expression in plants which synthesizes a PR-1α-heliomicine fusion

structure of this multiple cloning site is represented in Figure 4.

pRPA-RD-240: Introduction of the cassette for expression of PR-1α-heliomicine from pRPA-RD-239 into pRPA-RD-195

The plasmid pRPA-RD-239 is digested with the restriction enzyme PstII. The DNA fragment containing the cassette for expression of PR-1α-heliomicine is purified. The purified fragment is then ligated into pRPA-RP-195 which has been previously digested with the restriction enzyme PstII and dephosphorylated with calf intestinal phosphatase.

pRPA-RD-174: Plasmid derived from pRPA-BL-150A (EP 0,508,909) containing the gene for tolerance to bromoxynil of pRPA-BL-237 (EP 0,508,909)

The gene for tolerance to bromoxynil is isolated from pRPA-BL-237 by gene amplification by PCR. The fragment obtained is blunt-ended and is cloned into the EcoRI site of pRPA-BL-150A which has been made blunt-ended by the action of Klenow polymerase under standard conditions. An *Agrobacterium tumefaciens* vector is obtained which contains the gene for tolerance to bromoxynil near its right border, a gene for tolerance to kanamycin near its left border and a multiple cloning site between these two genes.

The schematic structure of pRPA-RD-174 is represented in Figure 5. In this figure, "nos"

plasmid pRPA-RD-174 is digested with the restriction enzyme XmaI, and the large DNA fragment is purified. The two DNA fragments obtained are then ligated.

A plasmid derived from pRPA-RD-174 is obtained which comprises other restriction sites between the gene for tolerance to bromoxynil and the selectable marker kanamycin gene.

The schematic structure of the plasmid pRPA-RD-184 is represented in Figure 6 where the terms "nos", "NPT-II", "NOS pro", 35S pro", "BRX gene", "RB" and "LB" have the same meaning as in Figure 5.

pRPA-RD-241: Creation of an *Agrobacterium tumefaciens* vector containing the construct of the gene encoding heliomicine directed towards the extracellular matrix

The plasmid pRPA-RD-240 is digested with the restriction enzymes SfiII and AscI and the DNA fragment containing the PR-1α-heliomicine gene is purified. The plasmid pRPA-RD-184 is digested with the same restriction enzymes. The DNA fragment containing the cassette for expression of PR-1α-heliomicine is then ligated into pRPA-RD-184. An *Agrobacterium tumefaciens* vector is thus obtained which contains the sequence encoding the PR-1α-heliomicine fusion protein which leads to the expression of heliomicine in the extracellular matrix of the plant.

Example IV-2: Creation of an expression cassette CsVMV promoter - PG1 signal peptide - heliomicine - Nos

pRPA-NP5: Creation of a sequence encoding heliomicine fused with the signal peptide of the PG1 gene

The region encoding heliomicine was amplified by PCR from the clone pRPA-PS-PR1α-helio (SEQ ID NO 3) with the thermostable Pfu enzyme (Stratagene) according to the standard conditions recommended by the manufacturer. The synthetic oligonucleotides used for this amplification are:

Oligo 17: 5' GATAAGCTTA TCGGTTCCTG CGTG 3'

Oligo 18: 5' GGCTCGAGTC AAGTCTCGCA CCAGCAGTTC AC 3'

The PCR product was digested with the restriction enzyme XhoI and cloned into the vector pRPA-NP4 digested with the restriction enzymes SfoI and XhoI. The resulting clone therefore comprises the region encoding the signal peptide of the PG1 gene fused in the same reading frame with the sequence encoding heliomicine (SEQ ID NO 18).

pRPA-NP6: Creation of a cassette for expression of heliomicine in a transformation vector

The expression and transformation vector pILTAB 357 is derived from the binary vector pBin19. It contains the CsVMV promoter (Verdaguer *et al.* 1996, Plant Mol. Biol. 31, 1129-1139) followed by a multiple cloning site and the nopaline synthase Nos transcription terminator (Figure X+1). The sequence of this fragment is indicated (SEQ ID NO 19).

The heliomicine expression vector was

shoots formed during this stage are then developed for 10 days by culturing on an MS medium supplemented with 30 g/l of sucrose but containing no hormone. Next, developed shoots are collected and they are cultivated on an MS rooting medium containing half the content of salt, vitamins and sugar and containing no hormone. After about 15 days, the rooted shoots are transferred into soil.

3.3- Analysis of the expression of heliomicine in transgenic tobacco

a) Production of specific polyclonal antibodies

Polyclonal antibodies were obtained by immunizing a rabbit with native heliomicine according to the usual procedures of the Centre de Bioexpérimentation VALBEX (IUT A - Lyon I). The serum obtained (15 ml) was then immunopurified on Sepharose 4B column (Pharmacia; ref 17-0430-01) coupled to heliomicine so as to specifically select the immunoglobulins which recognize this peptide. These antibodies were finally eluted in 6 ml of glycine (200 mM; pH 3), neutralized with 1 M Tris pH 9.5, dialysed with 0.5× PBS, and stored frozen at -20°C up to the time of use.

b) Immunodetection of heliomicine in transgenic tobacco

Analysis of the expression of heliomicine was

film (ECL).

The result of this experiment shows that 4 transgenic tobacco plants strongly express heliomicine. The signal in the other transgenic plants is weak or not significant compared with the wild-type control. The signal observed for the best plant is at the level of the positive control (50 ng of heliomicine), which indicates that in this plant, heliomicine represents by weight about 1% of the total proteins.

Example V-1: emulsifiable concentrates

Example EC1:

-active substance	400 g/l
-alkali metal dodecylbenzenesulphonate	24 g/l
-oxyethylated nonylphenol containing 10 molecules of ethylene oxide	16 g/l
-cyclohexanone	200 g/l
-aromatic solvent	qs 1 litre

Example EC2:

-active substance	250 g
-epoxidized vegetable oil	25 g
-mixture of alkylarylsulphonate and polyglycol ether and fatty alcohols	100 g
-dimethylformamide	50 g
-xylene	575 g

Example WP 3:

-active substance	75%
-wetting agent	1.50%
-dispersing agent	8%
-calcium carbonate (inert filler)	qs 100%

Example WP 4:

-active substance	90%
-ethoxylated fatty alcohol (wetting agent)	4%
-ethoxylated phenylethylphenol (dispersing agent)	6%

Example WP 5:

-active substance	50%
-mixture of anionic and nonionic surfactants (wetting agent)	2.5%
-sodium lignosulphonate (dispersing agent)	5%
-kaolinic clay (inert carrier)	42.5%

Example V-4: dispersible granules

Example DG 1

90% by weight of active substance and 10% of pearl urea are mixed in a mixer. The mixture is then ground in a toothed roll grinder. A powder is obtained which is wetted with about 8% by weight of water. The wet powder is extruded in a perforated roll extruder. Granules are obtained which are dried and then crushed and sieved so as to retain respectively only the

prepared:

- peptide heliomicine M 2	22.4 mg
- distilled water	qs 2 cm^3

Example VI. Stability of the activity of heliomicine

The stability of an antimicrobial peptide towards plant proteases is an essential factor for obtaining a good level of expression and therefore of resistance to phytopathogens in transgenic plants. This stability is for example a critical point for an insect antimicrobial peptide such as cecropin B (Owens and Heutte, 1997, MPMI vol 10, No. 4, pp 525-528). We examined the stability of heliomicine and of its activity on a test phytopathogen (Botrytis cinerea) after incubation with crude extracts of 8 major crop plants (maize, wheat, barley, rape, soyabean, sunflower, tomato and tobacco).

The leaves of these 8 species were ground at low temperature (liquid nitrogen) in a mortar, and then the powder was resuspended in the same volume of water. After centrifugation (10,000 g for 30 minutes), the supernatant was recovered and the protein concentration determined. This concentration was adjusted for the 8 extracts to 1 mg/ml by dilution with water and then these extracts were filtered sterilely (0.2 microns). One hundred microlitres of each extract (as well as a

chosen in order to introduce the mutations.

- **heliomicine R48**: replacement of the amino acid Glu48 of the sequence ID NO: 1 with a basic amino acid, in particular an arginine (Arg48). By analogy with the sequence encoding the heliomicine having the sequence: SEQ ID NO: 1, the codon GAA encoding Glu is replaced by the codon AGA encoding Arg. The oligonucleotides 19 and 20 are used as a replacement for the oligonucleotides 5 and 6 of Example II.

Oligo 19: 5′ GATCCTTCGC TAACGTTAAC TGTTGGTGTA GAACCTGATA GG 3′

Oligo 20: 5′ TCGACCTATC AGGTTCTACA CCAACAGTTA ACGTTAGCGA AG 3′

- **heliomicine L28L29**: replacement of two basic amino acids Lys and Arg at position 28 and 29 of the sequence ID NO: 1 with two hydrophobic amino acids, in particular two leucine amino acids (Leu28 and 28). By analogy with the sequence encoding the heliomicine having the sequence: SEQ ID NO:1, the part AAG-CGC encoding the peptide residue Lys-Arg is replaced by the sequence TTG-TTG encoding the peptide residue Leu-Leu. The oligonucleotides 21 and 22 are used as a replacement for the oligonucleotides 3 and 4 of Example II.

Oligo 21: 5′ CTAGTGACTG CAACGGCGAG TGCTTGTTGC GC 3′

Oligo 22: 5′ GCAACAAGCA CTCGCCGTTG CAGTCA 3′

- **heliomicine L28L29R48**: replacement of the

the oligonucleotides 27 and 28 as a replacement for the oligonucleotide 2.

Oligo 25: 5′ AGCTTGGATA AAAGAGCTGC TGCTGCTGGT AGCTGTGTTT 3′

Oligo 26: 5′ GGGGCGCCG TCAACTACA 3′

Oligo 27: 5′ CTAGTGTAGT TGACGGCGCC CC 3′

Oligo 28: 5′ AAACACAGCT ACCAGCAGCA GCAGCTCTTT TATCCA 3′

- heliomicine A24A25L28L29: Two oligonucleotides (sense and antisense) were necessary to compensate for the absence of a restriction site between the sequence encoding the peptide residue consisting of the two amino acids Asn24-Gly25 and the sequence encoding the peptide residue consisting of the two amino acids Lys28-Arg29 of the heliomicine of the sequence ID NO: 1. The two oligonucleotide sequences 29 and 30 replace respectively the two oligonucleotide sequences 3 and 4 of Example II.

Oligo 29: 5′ CTAGTGACTG CGCTGCTGAG TGCTTGTTGC GC 3′

Oligo 30: 5′ GCAACAAGCA CTCAGCAGCG CAGTCA 3′

Production of mutated heliomicine on the semipreparative scale

The various mutants of heliomicine are prepared and purified in the following manner. One of the transformed yeast clones expressing the mutated heliomicine was cultured at 29°C for 48 h in 50 ml of selective medium. This preculture was then used to inoculate 2 l of selective medium and the culture was

acetate, pH 3.4. The 1 M NaCl fraction containing the mutated heliomicine is recovered, dried under vacuum, reconstituted with 20 ml of acidified ultrapure water (1% TFA). The mutated heliomicine is then purified by reversed-phase HPLC.

- last purification step by HPLC: the mutated heliomicine was purified to homogeneity by chromatography on a preparative reversed-phase column Aquapo re RP-300 C8 (Brownlee™, 220 × 10 mm, 300 Å), using a biphasic linear gradient of acetonitrile from 2% to 23% over 10 min and from 23% to 33% over 80 min in 0.05% TFA at constant flow rate of 2.5 ml/min. The fraction collected is dried under vacuum, reconstituted with ultrapure water and analysed by MALDI mass spectrometry in order to verify the purity and the identity. The different mutated heliomicines were analysed for their antifungal activity under the conditions described for the reference heliomicine against the following strains: Neurospora crassa, Fusarium culmorum and Nectria haematococca. The activity of the mutants of heliomicine was also evaluated against bacteria. The experimental conditions used are described below.

Test of activity in vitro: measurement of the antibacterial and antifungal activity by microspectrophotometry

This methodology was used for the

Strasbourg), *Aerococcus viridans* (H. Monteil, Institute of bacteriology, Strasbourg), and *Escherichia coli D22* (P.L. Boquet, Centre for nuclear studies, Saclay).

Table 3: Activity of some mutated heliomicines against filamentous fungi and bacteria

Microorganisms	MIC for the mutants of heliomicine (μm)				
	L28L29	R48	L28L29R48	A6A7A8A9	Helio
Fungi					
Neurospora crassa	0.8-1.6	0.4-0.8	0.8-1.6	1.6-3.1	0.1-0.2
Fusarium culmorum	3.1-6.2	0.4-0.8	0.8-1.6	3.1-6.2	0.2-0.4
Nectria haematococca	3.1-6.2	0.4-0.8	0.8-1.6	ND	0.4-0.8
Bacteria					
Bacillus megaterium	50-100	ND	ND	6.2-12.5	ND
Micrococcus luteus	12.5-25	25-50	ND	ND	ND
Staphylococcus aureus	ND	ND	ND	ND	ND
Aerococcus viridans	ND	ND	ND	12.5-25	ND
Escherichia coli D22	ND	ND	ND	ND	ND

ND: no activity detected

CLAIMS

1. Peptide comprising essentially the peptide sequence of formula (I),

Xaa-Cys-Xab-Cys-Xac-Cys-Xad-Cys-Xae-Cys-Xaf-Cys-Xag (I)

in which:

Xaa is $-NH_2$ or a peptide residue comprising from 1 to 10 amino acids, preferably from 1 to 6 amino acids,

Xab is a peptide residue comprising from 1 to 10 amino acids, preferably 10,

Xac is a peptide residue of 3 amino acids,

Xad is a peptide residue comprising from 1 to 9 amino acids, preferably 9,

Xae is a peptide residue comprising from 1 to 7 amino acids, preferably 7,

Xaf is a peptide residue of 1 amino acid, and

Xag is -OH or a peptide residue comprising from 1 to 5 amino acids, preferably 1 or 2 amino acids.

2. Peptide according to claim 1, characterized in that

Xaa comprises at least one basic amino acid, and/or

Xad comprises at least one basic amino acid.

10. Peptide according to one of claims 1 to 10, characterized in that Xac represents the following peptide sequence -Asn-Gly-Glu-.

11. Peptide according to one of claims 1 to 10, characterized in that

Xaa represents the following peptide sequence Xaa'-Gly-Xaa"- in which Xaa' represents NH_2 or a peptide residue comprising 1 to 9 amino acids, preferably 1 to 5 amino acids, and Xaa" represents a peptide residue comprising at least one amino acid, preferably chosen from Leu, Ile, Val, Pro, Ser or Thr, and/or

Xab represents the following peptide sequence -Val-Xab'-Asp-, in which Xab' represents a peptide residue comprising from 0 to 8 amino acids, preferably 8, and/or

Xae represents the following peptide sequence -Gly-Xae'-Asn-, in which Xae' represents a peptide residue comprising from 0 to 5 amino acids, preferably 5, and/or

Xaf represents one of the following amino acids Trp, Phe, Leu, Ile or Val and/or

Xag represents the following peptide sequence -Glu-Xag' in which Xag' represents OH or a variable residue having a sequence comprising from 1 to 4 amino acids, preferably 1 amino acid.

12. Peptide according to one of claims 1 to 11, characterized in that

18. Fusion peptide according to claim 17, characterized in that the peptide fused with heliomicine is a signal peptide or a transit peptide.

19. Fusion peptide according to claim 18, characterized in that the transit peptide is the signal peptide of the tobacco PR-1α gene or the precursor of factor Mat alpha 1 or the signal peptide of the maize polygalacturonase PG1 gene.

20. Fusion peptide according to claim 19, characterized in that it is represented by the sequence identifier No. 1 (SEQ ID NO 1), by the sequence identifier No. 3 (SEQ ID NO 3), or by the sequence identifier No. 18 (SEQ ID NO 18).

21. As a medicament, the peptide according to one of claims 1 to 20.

22. Composition, characterized in that it comprises the peptide according to one of claims 1 to 20 and an appropriate vehicle.

23. Nucleic acid fragment, characterized in that it comprises a nucleic acid sequence encoding a peptide according to one of claims 1 to 20.

24. Nucleic acid fragment according to claim 23, characterized in that it is a nucleotide sequence of the DNA type.

30. Transformed host organisms, characterized in that they contain a nucleic acid fragment according to claims 23 to 25, or a chimeric gene according to claims 26 to 28.

31. Transformed host organism according to claim 30, characterized in that it includes microorganisms, plant cells or plants.

32. Transformed host organism according to claim 30, characterized in that it is a plant containing transformed cells.

33. Host organism according to claim 32, characterized in that the plant is regenerated from transformed cells.

34. Transformed host organism according to claim 30, characterized in that the microorganism is chosen from bacteria, in particular *E. coli*, yeasts, in particular of the genera *Saccharomyces* or *Kluyveromyces, Pichia*, fungi, in particular *Aspergillus*, or baculoviruses.

35. Transformed plant cell, characterized in that it contains a nucleic acid fragment according to claims 23 to 25 or a chimeric gene according to claims 26 to 28.

36. Transformed plant, characterized in that it comprises at least one transformed plant cell according to claim 35.

43. Method of cultivating transformed plants according to one of claims 36 to 38, characterized in that it consists in planting the seeds of the said transformed plants in a plot of a field appropriate for cultivating the said plants, in applying to the said plot of the said field an agrochemical composition, without substantially affecting the said seeds or the said transformed plants, then in harvesting the cultivated plants when they arrive at the desired maturity and optionally in separating the seeds from the harvested plants.

44. Method of cultivation according to claim 33, characterized in that the agrochemical composition comprises at least one active product having at least one fungicidal and/or bactericidal activity.

45. Method of cultivation according to claim 44, characterized in that the active product exhibits an activity which is complementary to that of the peptide according to one of claims 1 to 20.

46. Method of preparing heliomicine defined according to one of claims 1 to 20, characterized in that it comprises the steps of culturing a transformed organism according to one of claims 30 to 34 in an appropriate culture medium, followed by the extraction and total or partial purification of the heliomicine obtained.

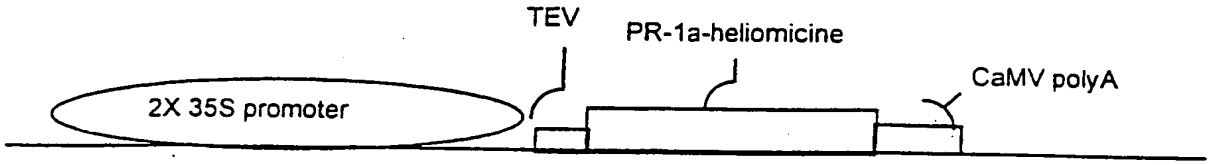

Fig. 3

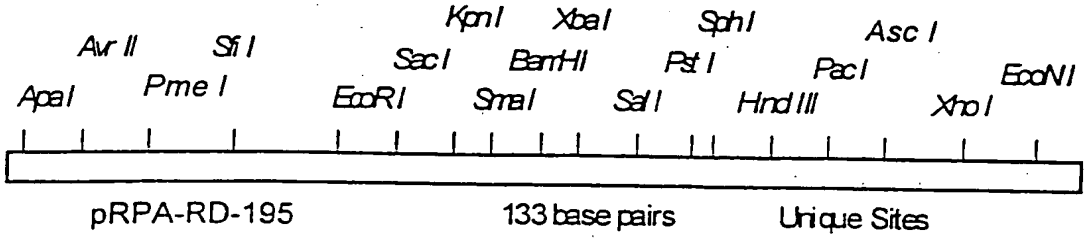

Fig. 4

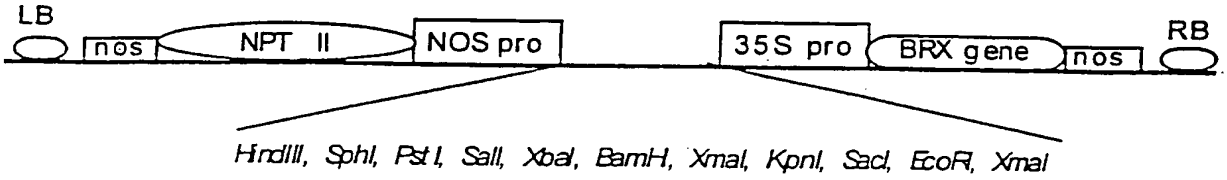

Fig. 5

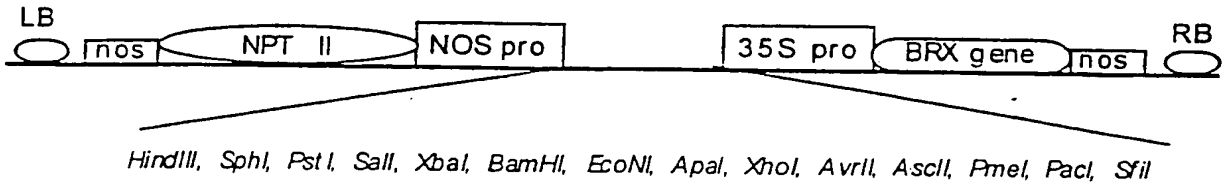

Fig. 6

(A) LENGTH: 147 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..147

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
AGC TTG GAT AAA AGA GAC AAG TTG ATT GGC AGC TGT GTT TGG GGC GCC      48
Ser Leu Asp Lys Arg Asp Lys Leu Ile Gly Ser Cys Val Trp Gly Ala
 1               5                  10                  15

GTC AAC TAC ACT AGT GAC TGC AAC GGC GAG TGC AAG CGC CGC GGT TAC      96
Val Asn Tyr Thr Ser Asp Cys Asn Gly Glu Cys Lys Arg Arg Gly Tyr
            20                  25                  30

AAG GGT GGC CAT TGT GGA TCC TTC GCT AAC GTT AAC TGT TGG TGT GAA     144
Lys Gly Gly His Cys Gly Ser Phe Ala Asn Val Asn Cys Trp Cys Glu
        35                  40                  45

ACC                                                                 147
Thr
 49
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 169 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
CC ATG GGT TTC GTG CTT TTC TCT CAG CTT CCA TCT TTC CTT CTT GTG          47
   Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu Leu Val
     1               5                  10                  15

TCT ACT CTT CTT CTT TTC CTT GTG ATC TCT CAC TCT TGC CGT GCC GAT         95
Ser Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg Ala Asp
                 20                  25                  30

AAG CTT ATC GGT TCC TGC GTG TGG GGT GCT GTG AAC TAC ACT TCC GAT        143
Lys Leu Ile Gly Ser Cys Val Trp Gly Ala Val Asn Tyr Thr Ser Asp
             35                  40                  45

TGC AAC GGT GAG TGC AAG AGG AGG GGT TAC AAG GGT GGT CAC TGC GGT        191
Cys Asn Gly Glu Cys Lys Arg Arg Gly Tyr Lys Gly Gly His Cys Gly
         50                  55                  60

TCC TTC GCT AAC GTG AAC TGC TGG TGC GAG ACT TGAGAGCTCG GCGAGGCGAA      244
Ser Phe Ala Asn Val Asn Cys Trp Cys Glu Thr
     65                  70

CGTGTCGACG GATCCGG                                                     261
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 120 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 12..101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 8"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA GAAGAGTAGA CACAAGAAGG 60

AAAGATGGAA GC 72

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 80 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 9"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GATAAGCTTA TCGGTTCCTG CGTGTGGGGT GCTGTGAACT ACACTTCCGA TTGCAACGGT 60

GAGTGCAAGA GGAGGGGTTA 80

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 109 base pairs

(B) TYPE: nucleotide

(A) LENGTH: 66 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 12"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC 60

TAGAGG 66

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 13"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC CCCGGCGCGC CTAGGTGTGT 60

GCTCGAGGGC CCAACCTCAG TACCTGGTTC AGG 93

(2) INFORMATION FOR SEQ ID NO: 12:

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 16"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCGAATTCGG CGCCGTGCAC GATGCAGAAG AGCACGAGGA GGAAGAGGGC 50

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 81 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 18"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGCTCGAGTC AAGTCTCGCA CCAGCAGTTC AC 32

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 213 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 7..205

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TCTAGA ATG GCC TGC ACC AAC AAC GCC ATG AGG GCC CTC TTC CTC CTC 48

(A) NAME/KEY: terminator

(B) LOCATION: 569..832

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AAGCTTCCAG AAGGTAATTA TCCAAGATGT AGCATCAAGA ATCCAATGTT TACGGGAAAA 60

ACTATGGAAG TATTATGTGA CCTCAGCAAG AAGCAGATCA ATATGCGGCA CATATGCAAC 120

CTATGTTCAA AAATGAAGAA TGTACAGATA CAAGATCCTA TACTGCCAGA ATACGAAGAA 180

GAATACGTAG AAATTGAAAA AGAAGAACCA GGCGAAGAAA AGAATCTTGA AGACGTAAGC 240

ACTGACGACA ACAATGAAAA GAAGAAGATA AGGTCGGTGA TTGTGAAAGA GACATAGAGG 300

ACACATGTAA GGTGGAAAAT GTAAGGGCGG AAAGTAACCT TATCACAAAG GAATCTTATC 360

CCCCACTACT TATCCTTTTA TATTTTTCCG TGTCATTTTT GCCCTTGAGT TTTCCTATAT 420

AAGGAACCAA GTTCGGCATT TGTGAAAACA AGAAAAAATT TGGTGTAAGC TATTTTCTTT 480

GAAGTACTGA GGATACAACT TCAGAGAAAT TTGTAAGTTT GTAGATCTCG ATTCTAGAAG 540

GCCTGAATTC GAGCTCGGTA CCGGATCCAA TTCCCGATCG TTCAAACATT TGGCAATAAA 600

GTTTCTTAAG ATTGAATCCT GTTGCCGGTC TTGCGATGAT TATCATATAA TTTCTGTTGA 660

ATTACGTTAA GCATGTAATA ATTAACATGT AATGCATGAC GTTATTTATG AGATGGGTTT 720

TTATGATTAG AGTCCCGCAA TTATACATTT AATACGCGAT AGAAAACAAA ATATAGCGCG 780

CAAACTAGGA TAAATTATCG CGCGCGGTGT CATCTATGTT ACTAGATCGG GGATCGAT 838

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1036 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CsVMV promoter

```
ATG GCC TGC ACC AAC AAC GCC ATG AGG GCC CTC TTC CTC CTC GTG CTC          586
Met Ala Cys Thr Asn Asn Ala Met Arg Ala Leu Phe Leu Leu Val Leu
 1               5                  10                  15

TTC TGC ATC GTG CAC GGC GAT AAG CTT ATC GGT TCC TGC GTG TGG GGT          634
Phe Cys Ile Val His Gly Asp Lys Leu Ile Gly Ser Cys Val Trp Gly
            20                  25                  30

GCT GTG AAC TAC ACT TCC GAT TGC AAC GGT GAG TGC AAG AGG AGG GGT          682
Ala Val Asn Tyr Thr Ser Asp Cys Asn Gly Glu Cys Lys Arg Arg Gly
        35                  40                  45

TAC AAG GGT GGT CAC TGC GGT TCC TTC GCT AAC GTG AAC TGC TGG TGC          730
Tyr Lys Gly Gly His Cys Gly Ser Phe Ala Asn Val Asn Cys Trp Cys
    50                  55                  60

GAG ACT TGACTCGAGG GGGGGCCCGG TACCGGATCC AATTCCCGAT CGTTCAAACA          786
Glu Thr
 65

TTTGGCAATA AAGTTTCTTA AGATTGAATC CTGTTGCCGG TCTTGCGATG ATTATCATAT      846

AATTTCTGTT GAATTACGTT AAGCATGTAA TAATTAACAT GTAATGCATG ACGTTATTTA      906

TGAGATGGGT TTTTATGATT AGAGTCCCGC AATTATACAT TTAATACGCG ATAGAAAACA      966

AAATATAGCG CGCAAACTAG GATAAATTAT CGCGCGCGGT GTCATCTATG TTACTAGATC      1026

GGGGATCGAT                                                             1036
```

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

ACTACACTAG TGACTGCAAC GGCGAGTGCA AGCGCCGCGG TTACAAGGGT GG 52

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CACAATGGCC ACCCTTGTAA CCGCGGCGCT TGCACTCGCC GTTGCAGTCA CT 52

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 56 base pairs

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 19"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GATCCTTCGC TAACGTTAAC TGTTGGTGTA GAACCTGATA GG 42

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 22"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GCAACAAGCA CTCGCCGTTG CAGTCA 26

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 23"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CTAGTGACTG CGCTGCTGAG TGCAAGCGGC GC 32

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

AGCTTGGATA AAAGAGCTGC TGCTGCTGGT AGCTGTGTTT 40

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 26"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GGGGCGCCGT CAACTACA 18

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 29"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CTAGTGACTG CGCTGCTGAG TGCTTGTTGC GC 32

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic oligonucleotide 30"

INTERNATIONAL SEARCH REPORT

International Application No
PCT/FR 99/00843

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHUNG K T ET AL: "Antibacterial factors in immune hemolymph from heliothis virescens larvae" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 96, no. 0, 19 May 1996 (1996-05-19), page 275 XP002089180 WASHINGTON US abstract	13,14,25
A	DE 22 12 854 A (WSESOJUSNYJ NAUTSCHNO) 2 November 1972 (1972-11-02) the whole document	1,21,22, 46
A	WO 97 30082 A (RHONE POULENC AGROCHIMIE) 21 August 1997 (1997-08-21) the whole document & FR 2 745 004 A cited in the application	1,21,22
A	EP 0 307 841 A (THE GENERAL HOSPITAL CORP.) 22 March 1989 (1989-03-22) the whole document	17,19, 20,23, 25,26, 29,30, 35,36
A	EP 0 607 080 A (TRANSGENE SA) 20 July 1994 (1994-07-20) page 2, line 28 - line 53	17-19
A	HOFFMANN J A ET AL.: "Insect defensins: inducible antibacterial peptides" IMMUNOLOGY TODAY., vol. 13, no. 10, 1992, pages 411-415, XP002089181 CAMBRIDGE GB the whole document	1-46
P,X	LAMBERTY M ET AL.: "Insect immunity - Isolation from the lepidopteran Heliothis virescens of a novel insect defensin with potent antifungal activity" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 274, no. 14, 2 April 1999 (1999-04-02), pages 9320-9326, XP002112857 BALTIMORE, US ISSN: 0021-9258 the whole document	1-25

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

RAPPORT DE RECHERCHE INTERNATIONALE

Dem: Internationale No
PCT/FR 99/00843

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 6 C12N15/12 C07K14/435 C12N15/82 A61K38/17 C12P21/02
C12N15/62 C12N15/81

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)
CIB 6 C07K C12N

Documentation consultee autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porte la recherche

Base de donnees electronique consultee au cours de la recherche internationale (nom de la base de donnees, et si realisable, termes de recherche utilises)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégone *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visees
X	FR 2 695 392 A (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE) 11 mars 1994 (1994-03-11) page 1, ligne 33 - page 7, ligne 2	1-4,8, 11,21, 22,46
Y	---	13,14,25
X	WO 90 11770 A (CALGENE INC) 18 octobre 1990 (1990-10-18) page 1 - page 19	1-4,8, 11,17, 18, 21-24, 28-46

X	FR 2 725 992 A (RHONE POULENC AGROCHIMIE) 26 avril 1996 (1996-04-26) cité dans la demande le document en entier	1-4,8, 11,21,22

	-/--	

[X] Voir la suite du cadre C pour la fin de la liste des documents

[X] Les documents de familles de brevets sont indiqués en annexe

* Catégones spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cite pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se referant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

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"T" document ultérieur publié après la date de depôt international ou la date de priorité et n'appartenenant pas à l'etat de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent: l'inven tion revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent: l'inven tion revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison etant évidente pour une personne du métier

"&" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée	Date d'expedition du present rapport de recherche internationale
27 août 1999	13/09/1999

Nom et adresse postale de l'administration chargee de la recherche internationale	Fonctionnaire autorisé
Office Européen des Brevets, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, A

Formulaire PCT/ISA/210 (deuxième feuille) (juillet 1992)

RAPPORT DE RECHERCHE INTERNATIONALE

Renseignements relatifs aux membres de familles de brevets

Demande internationale No
PCT/FR 99/00843

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
FR 2695392 A	11-03-1994	AUCUN	
WO 9011770 A	18-10-1990	CA 2030779 A	12-10-1990
		EP 0425616 A	08-05-1991
FR 2725992 A	26-04-1996	AUCUN	
DE 2212854 A	02-11-1972	CH 568387 A	31-10-1975
		FR 2130267 A	03-11-1972
		GB 1355163 A	05-06-1974
WO 9730082 A	21-08-1997	FR 2745004 A	22-08-1997
		AU 1884397 A	02-09-1997
		CA 2245518 A	21-08-1997
		CN 1216047 A	05-05-1999
		EP 0882063 A	09-12-1998
		PL 328579 A	01-02-1999
EP 0307841 A	22-03-1989	AU 2487788 A	17-04-1989
		WO 8902437 A	23-03-1989
EP 0607080 A	20-07-1994	FR 2700338 A	13-07-1994